

**METHODS OF BLOCKING B-CELL ACTIVATION USING
ANTI-CD40 MONOCLONAL ANTIBODIES**Cross-Reference to Related Applications

- 5 This application is a continuation-in-part of U.S. Serial No. 07/910,222, filed July 9, 1992, currently pending, the disclosure of which is hereby incorporated by reference.

Field of the Invention

- 10 This invention relates to novel methods of treating diseases of the immune system. In particular, this invention relates to methods of preventing or treating antibody-mediated diseases such as IgE-mediated disease (allergies) and autoimmune diseases including sytematic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), and idiopathic thrombocytopenic purpura (ITP).

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Background of the InventionI. B-Cell Activation

- 20 B cells play an important role during the normal *in vivo* immune response. A foreign antigen will bind to surface immunoglobulins on specific B cells, triggering a chain of events including endocytosis, processing, presentation of processed peptides on MHC-class II molecules, and up-regulation of the B7 antigen on the B-cell surface. A specific T cell then binds to the B cell via T-cell receptor (TCR) recognition of processed antigen presented on the MHC-class II molecule. Stimulation through the TCR begins to activate the T cell and initiates T-cell cytokine production. Interaction
25 between the CD28 antigen on T cells and the B7 antigen on B cells can provide a second signal further activating the T cell, resulting in high level cytokine secretion. Additionally, the CD40 ligand, which is not expressed on resting human T cells, is up-regulated on the T-cell surface when the above-mentioned signals are received. The B cell is then stimulated by the CD40 ligand through the CD40 antigen on the B-

cell surface, and also by soluble cytokines, causing the B cell to mature into a plasma cell secreting high levels of soluble immunoglobulin.

II. The EL4B5 Cell Line

5 A few years ago, Zubler et al., J. Immunol. (1985) 134:3662, observed that a mutant subclone of the mouse thymoma EL-4 line, known as EL4B5, could strongly stimulate B cells of both murine and human origin to proliferate and differentiate into immunoglobulin-secreting plasma cells *in vitro*. This activation was found to be antigen-independent and not MHC restricted. For optimal stimulation of human B
10 cells, the presence of supernatant from activated human T cells was needed, but a B-cell response also occurred when EL4B5 cells were preactivated with phorbol-12-myristate 13-acetate (PMA) or IL-1. Zubler et al., Immunological Reviews (1987) 99:281; and Zhang et al., J. Immunol. (1990) 144:2955. B-cell activation in this culture system is efficient -- limiting dilution experiments have shown that the
15 majority of human B cells can be activated to proliferate and differentiate into antibody-secreting cells. Wen et al. Eur. J. Immunol. (1987) 17:887.

The mechanism by which these mutant EL-4 cells activate both murine and human B cells has not been elucidated previously. It is, however, clear that cell-cell contact is required for EL4B5-induced B-cell activation. First, B cells do not
20 proliferate in the presence of supernatant from PMA-stimulated EL4B5 cells. Zubler et al. (1985) supra. Second, B cells do not proliferate when they are separated from PMA-treated EL4B5 cells by a semipermeable filter membrane. Zhang et al., supra. Antibodies against mouse LFA-1, human LFA-1 or human LFA-3 and antibodies against mouse or human MHC class II molecules do not inhibit EL4B5-induced
25 proliferation of human or murine B cells. Zubler et al. (1987) and Zhang et al., supra.

III. The CD40 Antigen, the CD40 Antigen Ligand, and Anti-CD40 Antibodies

The CD40 antigen is a glycoprotein expressed on the cell surface of B cells. During B-cell differentiation the molecule is first expressed on pre-B cells and then disappears from the cell surface when the B cell becomes a plasma cell. Crosslinking of the CD40 molecules with anti-CD40 antibodies mediates a variety of effects on B cells. The CD40 antigen is known to be related to the human nerve growth factor (NGF) receptor and tumor necrosis factor-alpha (TNF- α) receptor, suggesting that CD40 is a receptor for a ligand with important functions in B-cell activation.

A ligand for CD40 has been identified on the cell surface of activated T cells. Fenslow et al., J. Immunol. (1992) 149:655; Lane et al., Eur. J. Immunol. (1992) 22:2573; Noelle et al., Proc. Natl. Acad. Sci. (USA) (1992) 89:6550. cDNA cloning of the CD40 ligand revealed a molecule with characteristics of a type-II transmembrane glycoprotein with homology to TNF- α . Armitage et al., Nature (1992) 357:80 and Spriggs et al., J. Exp. Med. (1992) 176:1543. The extracellular domain of the CD40 ligand contains two arginine residues proximal to the transmembrane region, providing a potential proteolytic cleavage site that could give rise to a soluble form of the ligand. Expression of recombinant CD40 ligand has demonstrated that this molecule can stimulate the proliferation of purified B cells and, in combination with IL-4, mediate the secretion of IgE. Armitage et al. and Spriggs et al., supra. It has been reported that abnormalities in the gene for the CD40 ligand, resulting in the absence of a functional molecule on activated T cells, is responsible for the occurrence of X-linked hyper-IgM syndrome, a rare disorder characterized by the inability of these patients to produce normal levels of antibody isotypes other than IgM. Allen et al., Science (1993) 259:990; and Korthäuer et al., Nature (1993) 361:539.

All anti-CD40 antibodies known in the art have a stimulatory effect on human B cells. Cross-linking of the CD40 molecule on the B-cell surface using known anti-CD40 antibodies mediates a variety of effects on B cells. Anti-CD40 monoclonal antibodies (mAbs) can induce intercellular adhesion, proliferation and, in combination

with certain cytokines, maturation to antibody secreting cells. For example, known anti-CD40 mAbs have been shown to mimic the effects of T helper cells in B-cell activation. When presented on adherent cells expressing Fc γ RII, these antibodies induce B-cell proliferation. J. Banchereau et al., Science (1989) 251:70. Moreover, 5 the known anti-CD40 mAbs can replace the T helper signal for secretion of IgM, IgG and IgE in the presence of IL-4. H. Gascan et al., J. Immunol. (1991) 147:8. Furthermore, known anti-CD40 mAbs can prevent programmed cell death (apoptosis) of B cells isolated from lymph nodes.

However, the anti-CD40 antibodies known in the art stimulate B cells but are 10 incapable of inhibiting the B-cell response. Furthermore, no anti-CD40 antibodies are known that are (1) capable of inhibiting the B-cell response and (2) can be used to prevent or treat antibody-mediated disease.

15 Summary of the Invention

The current invention is based on the discovery of anti-CD40 antibodies that do not stimulate the growth and differentiation of human B cells. In contrast, these antibodies can inhibit human B-cell responses at relatively low concentrations. Accordingly, these antibodies can be used to prevent or treat diseases or conditions 20 that are mediated by antibodies produced by the human B-cell response. These antibodies also recognize novel epitopes on the CD40 antigen useful in modulating the B-cell response.

Accordingly, it is a primary object of this invention to provide a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a 25 human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell.

It is a further object of this invention to provide a method for preventing or treating an antibody-mediated disease in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount

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of a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient.

5 It is another object of this invention to provide a method for preventing or treating an IgE-mediated disease such as an allergy in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody
10 to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient.

It is yet another object of this invention to provide a method for preventing or treating an antibody-mediated autoimmune disease in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount
15 of a monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient. Particular autoimmune diseases contemplated for treatment by this method include sytematic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), and
20 idiopathic thrombocytopenic purpura (ITP).

It is a further object of this invention to provide a CD40 antigen epitope capable of competing with the binding of a CD40 antigen to an anti-CD40 monoclonal antibody wherein the binding of that antibody to a human CD40 antigen located on the surface of a human B cell prevents the growth or differentiation of the B cell.

25 In more preferred embodiments of the above objects, the monoclonal antibody is either 5D12, 3A8 or 3C6.

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Brief Description of the Drawings

Figure 1A shows a schematic representation of the baculoviral transfer vector pAcC8 and the sequence of the multiple cloning site. Figure 1B shows a schematic representation of the generation of Sf9 cells which express human CD40 or B7 antigen.

Figure 2 shows the sequences of polymerase chain reaction primers used in the preparation of coding regions for human CD40 and human B7 antigens. These primers were constructed on the basis of the published complete DNA coding sequences for antigens B7 and CD40.

Figure 3 shows the results of ELISA assays examining the reaction of anti-CD40 monoclonal antibody 52C6 with Sf9 cells expressing CD40 and with Sf9 cells expressing B7.

Figure 4 shows the results of the fluorescent cell staining of EBV-transformed B-cell line ARC cells expressing CD40.

Figure 5A compares the ability of three new and one old anti-CD40 mAbs to co-stimulate anti-IgM induced human B-cell proliferation. Figure 5B repeats the experiment of Figure 5A in the presence of recombinant interleukin-2 (rIL-2).

Figure 6 shows the ability of three new anti-CD40 mAbs to inhibit human B-cell proliferation induced by costimulation with immobilized anti-IgM and anti-CD40 mAb 52C6.

Figure 7 shows the effect of three new anti-CD40 mAbs on EL4B5-induced human B-cell proliferation.

Figure 8 shows the effect of soluble CD40 (hCD40. μ) on EL4B5-induced human B-cell proliferation.

Figures 9A and 9B show the effect of one new anti-CD40 mAb 5D12 on human T-cell induced immunoglobulin production by human B cells.

Detailed Description of the Invention

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and applications, cited previously or below are hereby incorporated by reference.

Definitions:

As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof such as F_{ab} , $F_{(ab)2}$, F_v , and other fragments which retain the antigen binding function of the parent antibody.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as F_{ab} , $F_{(ab)2}$, F_v , and others which retain the antigen binding function of the antibody. Monoclonal antibodies of any mammalian species can be used in this invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "humanized antibodies" means that at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences.

As used herein, the term "single chain antibodies" refer to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and

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construction of single chain antibodies are described in U.S. Patent No. 4,946,778 to Ladner et al.

The term "CD40 antigen epitope" as used herein refers to a molecule which is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40 antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (*i.e.*, organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, *inter alia*, in PCT application US91/04282.

The Antibody

The antibodies of the current invention bind to a human CD40 antigen on the surface of a human B cell and do not stimulate the growth of differentiation of the B cell. These antibodies may be polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof.

1. Antibody Preparation

Monoclonal antibodies 5D12, 3A8 and 3C6 are prepared as described in Example 1 herein. Other monoclonal antibodies of the invention may be prepared similarly, or as follows. First, polyclonal antibodies are raised against the CD40 antigen. Second, monoclonal antibodies specific for CD40 are selected.

a) Polyclonal Sera

Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally

performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 μ g/injection is typically sufficient. Immunization is generally boosted
5 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization.

Polyclonal antisera are obtained by bleeding the immunized animal into a glass
10 or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

b) Monoclonal Antibodies

15 Monoclonal antibodies are prepared using the method of Kohler and Milstein, Nature (1975) 256:495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) are removed and dissociated into single cells. If desired, the spleen cells may be screened (after
20 removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a
25 selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the desired immunizing cell-surface antigen (and which do not bind to unrelated antigens). The selected

mAb-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a mAb. Further, one may combine various labels for desired effect. For example, mAbs and avidin also require labels in the practice of this invention: thus, one might label a mAb with biotin, and detect its presence with avidin labeled with ^{125}I , or with an anti-biotin mAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

CD40 Antigen Epitopes

The CD40 antigen epitopes of this invention are molecules that are immunoreactive with anti-CD40 monoclonal antibodies whose binding to a human CD40 antigen located on the surface of a human B cell prevents the growth or differentiation of the B cell. That is, such epitopes compete with the binding of said antibodies to the CD40 antigen. Systematic techniques for identifying these epitopes

are known in the art, as described by H.M. Geysen in U.S. Patent No. 4,708,871, which is incorporated herein by reference. Typically these epitopes are short amino acid sequences. These sequences may be embedded in the sequence of longer peptides or proteins, as long as they are accessible.

5 The epitopes of the invention may be prepared by standard peptide synthesis techniques, such as solid-phase synthesis. Alternatively, the sequences of the invention may be incorporated into larger peptides or proteins by recombinant methods. This is most easily accomplished by preparing a DNA cassette which encodes the sequence of interest, and ligating the cassette into DNA encoding the
10 protein to be modified at the appropriate site. The sequence DNA may be synthesized by standard synthetic techniques, or may be excised from the phage pIII gene using the appropriate restriction enzymes.

Epitopes identified herein may be prepared by simple solid-phase techniques. The minimum binding sequence may be determined systematically for each epitope by
15 standard methods, for example, employing the method described by H.M. Geysen, U.S. Pat. No. 4,708,871. Briefly, one may synthesize a set of overlapping oligopeptides derived from the CD40 antigen bound to a solid phase array of pins, with a unique oligopeptide on each pin. The pins are arranged to match the format of
20 a 96-well microtiter plate, permitting one to assay all pins simultaneously, e.g., for binding to an anti-CD40 monoclonal antibody. Using this method, one may readily determine the binding affinity for every possible subset of consecutive amino acids.

Analogous of the invention are also prepared by standard solid-phase methods, and those methods described in PCT application US91/04282.

25 Formulations and Methods of Administration

The antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat antibody-mediated diseases such as allergies, SLE, PBC and ITP. To accomplish this goal, the antibodies may be formulated using a variety of acceptable excipients known in the art. Typically, the

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antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

- 5 Before administration to patients, formulants may be added to the antibodies. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as
- 10 mono, di, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having an -OH
- 15 group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0
- 20 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before
- 25 lyophilization or after reconstitution. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al., 1988, *J. Bio. Chem.* 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon

et al., Cancer Research (1982) 42:4734; Cafiso, Biochem Biophys Acta (1981) 649:129; and Szoka, Ann Rev Biophys Eng (1980) 9:467. Other drug delivery systems are known in the art and are described in , e.g., Poznansky et al., DRUG DELIVERY SYSTEMS (R.L. Juliano, ed., Oxford, N.Y. 1980), pp. 253-315; M.L.

5 Poznansky, Pharm Revs (1984) 36:277.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled
10 water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

As stated above, the antibodies and compositions of this invention are used to treat human patients to prevent or treat antibody-mediated diseases such as allergies,
15 SLE, PBC and ITP. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are saline, Ringer's solution,
20 dextrose solution, and Hanks' solution. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. A preferred vehicle is 5% dextrose in saline. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The dosage and mode of administration will depend on the individual.
25 Generally, the compositions are administered so that antibodies are given at a dose between 1 μ g/kg and 20 mg/kg, more preferably between 20 μ g/kg and 10 mg/kg, most preferably between 1 and 7 mg/kg. Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If so, the antibodies may

be infused at a dose between 5 and 20 $\mu\text{g/kg/minute}$, more preferably between 7 and 15 $\mu\text{g/kg/minute}$.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Examples

Materials and Methods

10 Cell Lines

The mutant mouse thymoma EL-4 subclone EL4B5 was a gift of Dr. R.H. Zubler, Hôpital Cantonal Universitaire, Geneva. Mouse 3T6 transfectant cells expressing hybrid molecules of the HR (high responder) allelic form of human Fc γ RIIa were a gift of Dr. P.A.M. Warmerdam, Department of Experimental Immunology, University Hospital Utrecht, Utrecht, The Netherlands. Warmerdam et al., J. Immunol. (1991) 147:1338. Both cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with gentamycin (80 $\mu\text{g/ml}$) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, Utah). To avoid possible loss of B cell activating capacity, every 4 to 8 weeks a new batch of EL4B5 cells was thawed. The cell lines were periodically tested for mycoplasma contamination by the use of a ^3H -labelled DNA probe for mycoplasma ribosomal RNA (GenProbe, San Diego, CA) and were free of mycoplasma during the course of the experiments.

Antibodies and hCD40.H μ Fusion Protein

25 Anti-CD40 mAb 5D12, 3C6 and 3A8 were generated by immunizing mice with insect cells expressing recombinant human CD40 as shown in Example 1. Anti-(B7) mAb B7-24 was generated in a similar way by immunizing with insect cells expressing recombinant human B7. Anti-CD40 mAb S2C6 was a gift of Dr. S. Paulie (University of Stockholm, Sweden). Paulie et al., J. Immunol. (1989)

142:590. Anti-CD40 mAb G28.5 was donated by Dr. J.A. Ledbetter (Oncogen Corporation, Seattle, WA, USA). Clark et al., PNAS (USA) (1986) 83:4494. Control antibodies were: anti-(β -glucocerebrosidase) mAb 8E4 (IgG1), Barneveld et al., Eur. J. Biochem. (1983) 134:585, and myeloma immunoglobulins MOPC-21 (IgG1) and MOPC-141 (IgG2b) (Sigma, St. Louis, MO). All mAb were used as purified antibody preparations. hCD40.H μ fusion protein was a gift of Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland) and was used as a 5x concentrated supernatant of transfected J558L cells. Lane et al., Eur. J. Immunol. (1992) 22:2573.

Human B Lymphocytes

B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomies, essentially as described in De Groot et al., Lymphokine Research (1990) 9:321. Briefly, the tissue was dispersed with scalpel blades, phagocytic and NK cells were depleted by treatment with 5 mM L-leucine methyl ester and T cells were removed by one cycle of rosetting with sheep erythrocytes (SRBC) treated with 2-aminoethyl isothiuronium bromide. The purity of the resulting B lymphocyte preparations was checked by indirect immunofluorescent labelling with anti-(CD20) mAb B1 (Coulter Clone, Hialeah, FA) or anti-(CD3) mAb OKT3 (Ortho, Raritan, NJ) and a FITC-conjugated F(ab')₂ fragment of rabbit anti-(mouse Ig) (Zymed, San Francisco, CA), and FACS analysis. The B cell preparations contained (mean \pm SD of 6 isolations): 95 \pm 4% CD20-positive cells and 2 \pm 1% CD3-positive cells.

B-Cell Proliferation Assay

B cells (4 x 10⁴ per well) were cultured in 200 μ l IMDM supplemented with 10% fetal calf serum in flat bottom 96-well microtiter plates. B cells were stimulated by addition of immobilized anti-(IgM) antibodies (Immunobeads; 5 μ g/ml; BioRad, Richmond, CA). Where indicated 100 U/ml recombinant IL-2 was added. Varying concentrations of mAbs were added at the onset of the microcultures and proliferation

was assessed at day 3 by measurement of the incorporation of [³H]-thymidine after 18 hour pulsing.

Banchereau-Like B-Cell Proliferation Assay

5 For testing the ability of anti-CD40 mAbs to stimulate B-cell proliferation in a culture system analogous to that described by Banchereau et al., Science (1989) 251:70, mouse 3T6 transfectant cells expressing the HR allelic form of human FcγRII were used. B cells (2 x 10⁴ per well) were cultured in flat-bottom microwells in the presence of 1 x 10⁴ transfectant cells (irradiated with 5000 Rad) in 200 μl
10 IMDM supplemented with 10% fetal calf serum and 100 U/ml recombinant IL-4. Before addition of the B cells, the 3T6 cells were allowed to adhere to the culture plastic for at least 5 hours. Anti-CD40 mAbs were added at concentrations varying from 15 ng/ml to 2000 ng/ml and proliferation of B cells was assessed by measurement of thymidine incorporation at day 7, upon 18 hour pulsing with [³H]-
15 thymidine.

B-Cell Activation Assay with EL4B5 Cells

B cells (1000 per well) were cultured together with irradiated (5000 Rad) EL4B5 cells (5 x 10⁴ per well) in flat bottom microtiter plates in 200 μl IMDM
20 supplemented with 10% heat-inactivated fetal calf serum, 5 ng/ml phorbol-12-myristate 13-acetate (Sigma) and 5% human T-cell supernatant. MAbs were added at varying concentrations at the onset of the cultures and thymidine incorporation was assessed at day 6 after 18 hour pulsing with [³H]-thymidine. For the preparation of T-cell supernatant, purified T cells were cultured at a density of 10⁶/ml for 36 hours
25 in the presence of 1 μg/ml PHA and 10 ng/ml PMA. Wen et al., supra. T-cell supernatant was obtained by centrifugation of the cells and stored at -20°C. The effectiveness of T-cell supernatants in enhancing proliferation of human B cells in EL4B5-B cell cultures was tested and the most effective supernatants were pooled and used in the experiments.

Human T Cell Helper Assay for Antibody Production by B Cells

96-well tissue culture plates were coated with a 1:500 dilution of ascites fluid of anti-CD3 mAb CLB-T3/3 (CLB, Amsterdam, The Netherlands). As indicated costimulatory mAbs were added: anti CD2 mAbs CLB-T11.1/1 and CLB-T11.2/1 (CLB, Amsterdam, The Netherlands), both ascites 1:1000 and anti-CD28 mAb CLB-28/1 (CLB, Amsterdam, The Netherlands). Subsequently, tonsillar T cells (irradiated, 3000 Rad; 10^5 per well), tonsillar B cells (10^4 per well) and rIL-2 (20 U/ml) were added. The final volume of each cell culture was 200 μ l. After 8 days, cells were spun down, and cell-free supernatant was harvested. The concentrations of human IgM and IgG in (diluted) samples were estimated by ELISA as described below.

ELISA Assay for Immunoglobulin Quantification

The concentrations of human IgM and IgG were estimated by ELISA. 96-well ELISA plates were coated with 4 μ g/ml mouse anti-human IgG mAb MH 16-01 (CLB, Amsterdam, The Netherlands) or with 1.2 μ g/ml mouse anti-human IgM mAb 4102 (Tago, Burlingame, CA) in 0.05 M carbonate buffer (pH = 9.6), by incubation for 16 h at 4°C. Plates were washed 3 times with PBS-0.05 % Tween-20 (PBS-Tween) and saturated with BSA for 1 hour. After 2 washes the plates were incubated for 1 h at 37°C with different dilutions of the test samples. After 3 washes, bound Ig was detected by incubation for 1 h at 37°C with 1 μ g/ml peroxidase-labeled mouse anti-human IgG mAb MH 16-01 (CLB) or mouse anti-human IgM mAb MH 15-01 (CLB). Plates were washed 4 times and bound peroxidase activity was revealed by the addition of O-phenylenediamine as a substrate. Human standard serum (H00, CLB) was used to establish a standard curve for each assay.

Flow Cytofluorometric Assay

ARC cells (10^6 cells/sample) were incubated in 100 μ l primary antibody (10 μ g/ml in PBS-BSA or Hanks' balanced salt solution (HBSS) supplemented with 1 %

BSA and 0.05 % sodium azide) for 20 min at 4°C. After 3 washes with PBS-BSA or HBSS-BSA, the cells were incubated in 100 µl FITC-labeled F(ab')₂ fragments of goat anti-(mouse IgG) antibodies (Jackson, West Grove, PA) for 20 min at 4°C. After 3 washes with PBS-BSA or HBSS-BSA and 1 wash with PBS, the cells were
5 resuspended in 0.5 ml PBS. Analyses were performed with a FACSCAN V (Becton Dickinson, San Jose, CA).

Alternatively, EL4B5 cells were harvested before and at different time points during culture in medium containing PMA (5ng/ml) and human T-cell supernatant (5%). Cells were incubated for 30 minutes with 10 µl supernatant of transfected cells
10 containing hCD40-Hµ diluted in 100 µl Hank's Balanced Salt Solution supplemented with 0.05 % sodium azide (4°C). This was followed by incubation with FITC-conjugated F(ab')₂ fragments of rabbit anti-(human IgM) (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). As a control, cells were incubated with the FITC-conjugate only. For analysis a FACScan-4 cytofluorometer
15 (Becton and Dickinson) was used. Non-vital cells were excluded from analysis by the use of propidium iodide.

Example 1

Making Monoclonal Antibodies to B7 and CD40

20 A. PCR Cloning of CD40 and B7

RNA was isolated from a population of EBV-transformed human spleen cells essentially as described by Chirgwin et al., Biochemistry (1979) 17:5294. In brief, the cells were washed twice with phosphate buffered saline (PBS) and lysed in 5 M guanidinium thiocyanate in the presence of 0.7 M 2-mercaptoethanol. The cell lysate
25 was layered on a discontinuous CsCl gradient (Chirgwin et al.) and centrifuged for 16 hours at 26,000 rpm in a Beckman SW28 rotor. The RNA was recovered by dissolving the pellet in DEPC-treated H₂O. The RNA was precipitated with ethanol once, resuspended in DEPC-treated H₂O, and stored at -70°C.

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0001504924550

Total RNA (10 μ g/reaction) was converted to cDNA using random hexamer priming in 50 μ l reaction buffer containing 500 units MLV-RT (Bethesda Research Laboratories, Bethesda, MD), 5 μ M random hexamer (Pharmacia, Piscataway, NJ), 1 mM DTT, dNTP mix (0.5 mM each), 10 mM Tris-HCL pH 8.3, 50 mM KCl, 2.5 mM MgCl₂ and 0.1 mg/ml BSA (bovine serum albumin). After incubation at 37°C for 1 hour, the samples were boiled for 3 minutes and stored at -70°C. The DNA encoding the CD40 and B7 molecules was generated by PCR using primers which contained sequences having homology to known CD40 and B7 sequence, where the primers also encoded restriction sites useful for cloning (Figure 2). These primers were based on the published cDNA coding sequences for B7 and CD40. Freeman et al., J. Immunol. (1989) 143:2714, and Stamenkovic et al., EMBO J. (1989) 8:1403. All primers start with a C-G clamp at the 5' end followed by a restriction site for cloning (shown in bold, Figure 2). The underlined sequences in the backward primers, for the cloning of the soluble forms of B7 and CD40, represents an epitope recognized by a monoclonal antibody used for affinity purification. The numbers in brackets represent the location of the primers relative to the published cDNAs for CD40 and B7.

For PCR amplification, 1 μ l of cDNA was mixed with 1 μ l (10 picomoles) of a forward primer, 1 μ l (10 picomoles) of a backward primer, and 47 μ l of PCR mix. The PCR mix consisted of 1.25 units Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), dNTP mix (0.2 mM each), 10 mM Tris-cHL pH 8.3, 50 mM KCl, 2.5 mM MgCl₂ and 0.1 mg/ml BSA. The 50 μ l of PCR mixture was overlaid with 70 μ l mineral oil and subjected to 25 cycles of amplification in a Perkin-Elmer/Cetus thermocycler (denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 1.5 minutes). PCR products were obtained after 25 amplification cycles.

The amplification products were digested with *Bgl*II and *Kpn*I (Figure 1B) and isolated by size-fractionation. Before expression in baculovirus, the DNA sequence of each fragment was confirmed by sequencing analysis to prevent the introduction of

PCR-induced mutations. The baculovirus transfer vector pAcC8 was also digested with *Bgl*III and *Kpn*I (Figure 1B).

The amplified fragments were ligated to the linear pAcC8 vector (ratio of insert to vector was 3:1). The ligation products were transformed into bacterial strain
5 DH5 α (Gibco/BRL, Gaithersburg MD) and recombinant pAcC8 vectors were selected on the basis of ampicillin resistance. Recombinant plasmids were isolated from bacterial clones (Maniatis et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories), 1982; Ausubel et al., Current Protocols in Molecular Biology (Media, PA: John Wiley and Sons)) and the
10 presence of the insert of interest verified using polymerase chain reactions (see above). Large scale plasmid preparation was performed by standard procedures (Ausubel et al.; Maniatis et al.; Sambrook et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories), 1989).

15 B. Baculovirus Expression of Human CD40 and B7

Sequences encoding human CD40 and human B7 were recombined into the *Autographa californica* baculovirus (AcNPV) using the transfer vectors pAcCD40 (encoding the full-length CD40 molecule), pAcCD40-ED/Glu (encoding the extracellular domain of CD40), pAcB7 (encoding the full-length B7 molecule) and
20 pCCb7-ED/Glu (encoding the cellular domain of the B7 molecule).

The plasmids were cotransfected with wild-type baculoviral DNA (2-10 pfu) (AcNPV; Summers et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987)) into Sf9 (*Spodoptera frugiperda*) cells at a density of 10⁶ cells/ml (Summers et
25 al.). Recombinant baculovirus-infected Sf9 cells were identified and clonally purified (Summers et al.).

For cell surface expression of recombinant proteins the cells were harvested after 48 hours of culture.

C. Sf9 Insect Cell ELISA

Sf9 insect cells infected with recombinant virus were cultured for 48 hours in 24-well plates. After removal of the tissue culture medium the plates were incubated for 45 minutes at room temperature (RT) with 0.25 ml of antibody in PBS with 1 %
5 BSA (PBS-BSA). After three washed with PBS-BSA, the plates were incubated for 35 minutes at RT with 250 μ l of a 1/250 dilution of goat anti-(mouse total Ig) immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) in PBS-BSA. Unbound peroxidase activity was removed by washing five times with PBS-BSA. Bound peroxidase activity was revealed by the addition of an assay
10 mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM sodium acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H_2O_2 . The reaction was stopped after 10 minutes by adding 100 μ l of 1 M H_2SO_4 .

The above-described ELISA assays performed on live Sf9 cells gave the
15 following results. Figure 3 presents the data for live Sf9 cells infected with pAcB7 and pAcCD40 which were cultured for 48 hours in 24-well plates. The antibodies used in the ELISA were: S2C6 (anti-CD40, open bars) and no primary antibody (hatched bars).

20 D. Host Animal Immunization

Female BALB/c mice were injected intraperitoneally at day 0 and day 14 with 5×10^6 Sf9 cells infected with AcCD40 virus, AcB7 virus or AcCd3 virus (control virus). At day 21, 100 μ l of serum was obtained to test for the presence of specific
25 antibodies. After a rest period of at least two weeks, the mice received a final injection with 5×10^6 cells infected with AcCD40 or AcB7 virus. Three days after this last injection, the spleen cells were used for cell fusion.

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E. Generation of Hybridoma Clones

Splenocytes from immunized BALB/c mice were fused with SP2/0 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer et al., J. Immunol. Meth. (1988) 113:143. The fused cells
5 were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, MA). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybrid on average.

- 10 After 10-14 days the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants of 12 wells were pooled and used for fluorescent cell staining of EBV-transformed B cells as described for the FACS Assay above. Subsequently, the supernatants of the positive pools were tested individually.
- 15 Positive hybridoma cells were cloned three times by limiting dilution in IMDM/FBS containing 0.5 ng/ml hIL-6. Three hybridomas producing anti-CD40 antibodies are labelled 5D12, 3A8 and 3C6. The data is presented in Figure 4, which shows that a soluble form of CD40, but not of B7 can block the binding of the anti-CD40 mAb 5D12 to CD40 expressing EBV-transformed B cells. Figure 4 shows fluorescent cell
- 20 staining of ARC EBV-transformed B cells with 5D12 in the presence and absence of soluble B7 and soluble CD40. 5D12 and the soluble B7, soluble CD40, or controls were preincubated at RT for 20 min before addition to the ARC cells. Figure 4A shows staining with 5D12 (dotted line) or second antibody only (solid line), figure 4B shows staining with 5D12 alone (dotted line) or preincubated with soluble B7 (solid
- 25 line) and, figure 4C shows staining with 5D12 alone (dotted line) or preincubated with soluble CD40.

Example 2Costimulation of B-Cell Proliferation Using Anti-CD40 mAbs

Four hybridomas producing monoclonal antibodies against human CD40 were generated in Example 1. These mAbs were shown to bind to a similar proportion of tonsillar B cells as anti-CD40 mAb G28.5 does. de Boer et al. J. Immunol. Methods (1992) 152:15. Three of these monoclonal antibodies (5D12, 3A8 and 3C6) which were of the IgG2b subclass, were tested for their ability to deliver activation signals to human B cells in the B-cell proliferation assay described above.

Human tonsillar B cells (4×10^4 per well) were cultured in 200 μ l in microwells in the presence of anti-IgM coupled to Sepharose beads (5 μ l/ml) (Figure 5A) or in the presence of anti-IgM plus rIL-2 (100 U/ml) (Figure 5B). Varying concentrations of the anti-CD40 mAbs S2C6, 5D12, 3C6 or 3A8 were added and [3 H]thymidine incorporation was measured at day 3 after 18 h pulsing. Data presented in Figure 5A are means derived from experiments with B-cell preparations from three different donors with duplicate incubations. Data of Figure 5B are means of duplicate incubations from one experiment out of two with comparable results.

None of the novel anti-CD40 mAbs was able to significantly costimulate human B-cell proliferation in the presence of immobilized anti-IgM or in the presence of immobilized anti-IgM and IL-2. In contrast, anti-CD40 mAb S2C6 costimulated human B-cell proliferation in a concentration dependent fashion.

Example 3Induction of B-Cell Proliferation Using Anti-CD40 mAbs

The mAbs tested in Example 2 were tested for their ability to induce proliferation of human B cells in the Banchereau-like Assay described above, i.e., by presenting the anti-CD40 mAb on adherent cells expressing Fc γ RII. As antibody presenting cells, mouse 3T6 transfectant cells expressing the HR allelic form of human Fc γ RII were used. It was observed that anti-CD40 mAb S2C6 together with IL-4 induced substantial proliferation of tonsillar human B cells in this system, as

assessed by measurement of [³H]thymidine incorporation. Anti-CD40 mAbs 5D12, 3C6 or 3A8 however, did not induce proliferation of human B cells in this culture system (data not shown).

5

Example 4

Inhibition of S2C6 Stimulated B-Cell Proliferation Using Anti-CD40 mAbs

The mAbs were also tested for their ability to inhibit the costimulation of human B-cell proliferation by anti-CD40 mAb S2C6 using the B-cell Proliferation Assay described above. Human tonsillar B cells (4×10^4 per well) were cultured in 10 200 μ l in microwells in the presence of anti-IgM coupled to Sepharose beads (5 μ g/ml) and anti-CD40 mAb S2C6 (1.25 μ g/ml). Varying concentrations of anti-CD40 mAbs 5D12, 3C6 or 3A8 were added and [³H]thymidine incorporation was assessed after 3 days. As a control anti-(glucocerebrosidase) mAb 8E4 was added in similar concentrations. Barneveld et al. Eur. J. Biochem. (1983) 134:585. Data are means 15 \pm S.D. derived from experiments with B cells from two different donors with duplicate incubations.

It was found that each of the anti-CD40 mAbs 5D12, 3A8 and 3C6 could inhibit the costimulation of anti-IgM induced human B-cell proliferation by mAb S2C6 (Figure 6). In contrast, no significant inhibition was seen with equivalent 20 amounts of non-relevant mAb 8E4, directed to β -glucocerebrosidase. Barneveld et al., supra. Thus, it was concluded that these anti-CD40 mAbs do not deliver stimulatory signals to the proliferation of human B cells, but, conversely, can inhibit stimulatory signals exerted by triggering anti-CD40 with another mAb. Therefore, these mAbs were considered to be excellent tools to investigate whether signaling via 25 CD40 plays a role in the stimulation of human B-cell proliferation by EL4B5 cells.

Example 5Effects of Anti-CD40 mAbs on EL4B5-Induced Human B-Cell Proliferation

The effect of anti-CD40 mAbs on EL4B5-induced human B-cell proliferation was tested using the B-cell Activation Assay described above. Human tonsillar B cells (1000 per well) were cultured together with irradiated EL4B5 cells (50,000 per well) in the presence of 5% supernatant of activated human T cells and 5 ng/ml PMA. Anti-CD40 mAbs 5D12, 3C6 or 3A8 were added in varying concentrations. As a control, mAb MOPC-141 (IgG2b) was added. After six days of culture, [³H]thymidine incorporation was assessed.

Figure 7 shows that addition of anti-CD40 mAbs 5D12, 3C6 or 3A8 resulted in a concentration-dependent inhibition of human B-cell proliferation. Data are means \pm S.D. derived from experiments with B cells from four different donors with duplicate incubations. [³H]-thymidine incorporation values found for incubations without mAb were (means \pm S.D.) 10460 \pm 1843 cpm, 6982 \pm 1729 cpm, 4362 \pm 1020 cpm and 1543 \pm 3190 in the four different experiments, respectively. [³H]-thymidine incorporation in B cells alone amounted to 40 \pm 5 cpm and in irradiated EL4B5 cells alone 31 \pm 15 cpm.

Very potent inhibition occurred. At concentrations as low as 10 ng/ml each, the three anti-CD40 mAbs 5D12, 3C6 and 3A8 inhibited human B-cell proliferation completely. Half-maximal inhibition was found at about 1 ng/ml. In contrast, isotype matched IgG2b mouse myeloma protein MOPC-141 had no significant effect on [³H]-thymidine incorporation. Similar inhibition was observed when [³H]-thymidine incorporation was assessed at day 4 of the culture instead of day 6, thus excluding the possibility that the observed effect was due to a change in the kinetics of the proliferation under influence of the anti-CD40 mAb (data not shown).

For comparison, the influence of a few mAb directed against other B-cell surface structures was investigated. Neither anti-CD20 mAb B1 or anti-B7 mAb B7-24 (the latter mAb was generated by a procedure similar to that used for generating the anti-CD40 mAb used in Figure 7, in concentrations similar to those used in the

experiments with the anti-CD40 mAb, had any effect on EL4B5-induced human B-cell proliferation (data not shown). Therefore, it may be concluded that the inhibitory effect of anti-CD40 mAb on EL4B5-induced B-cell proliferation is not due to masking of the B-cell surface.

5

Example 6

Effects of hCD40.H μ on EL4B5-Induced Human B-Cell Proliferation

In order to investigate whether EL4B5 cells expressed a membrane structure which binds CD40, a fusion protein consisting of the extracellular domain of CD40 and human IgM constant domains CH₂, CH₃ and CH₄ (hCD40.H μ) was used for flow fluorocytometric analysis. Lane et al., *supra*. Non-activated EL4B5 cells did not bind the fusion protein. However, upon culturing EL4B5 cells together with PMA (5 ng/ml) and 5% human T-cell supernatant, which are the conditions needed for activation of human B cells, a low binding of hCD40.H μ was found (data not shown). This small shift in fluorescence was found consistently in three independent experiments. The minimal activation period needed for induction of the CD40 binding was 24 hours. To determine whether binding of hCD40.H μ to the EL4B5 cells would inhibit EL4B5-induced human B-cell proliferation like anti-CD40 mAb did, the fusion protein was titrated into cocultures of EL4B5 cells with human B cells using the B-cell Activation Assay described above. Figure 8 shows that the fusion protein did indeed inhibit [³H]-thymidine incorporation in a concentration-dependent manner and, like the anti-CD40 mAb used in the experiments shown in Figure 7, was able to inhibit B-cell proliferation induced by the EL4B5 cells completely.

25

Example 7

Effects of Anti-CD40 mAbs on Human T-Cell-Induced Antibody Production by Human B-Cells

The antibodies were also tested for their capacity to inhibit immunoglobulin production by B cells, stimulated in a contact-dependent manner with activated T cells

using the T-cell helper assay described above. Human tonsillar B cells (10^4 /well) were cultured together with irradiated purified T cells (3000 rad, 10^5 /well) in 96-well plates, coated with anti-CD3 mAb and with or without different mAbs to costimulate the T cells. After 8 days of culture the supernatants were harvested for the

5 determination of immunoglobulin production by the B cells. Immunoglobulin production by the B cells was assessed by the ELISA assay described above. Anti-CD40 mAb 5D12 was added in varying concentrations from the onset of the cultures. As a control, mAb MOPC-141 was added. Figure 9A shows that when T cells were stimulated with immobilized anti-CD3 mAb and costimulated with soluble anti-CD2

10 and anti-CD28 mAbs, addition of anti-CD40 mAb 5D12 resulted in a concentration dependent inhibition of IgG production by human B cells. IgM production by the B cells was inhibited to the same extent. Similar results were obtained with the anti-CD40 mAbs 3C6 and 3A8 and with the hCD40.H μ fusion protein.

The anti-CD40 mAbs of this invention exhibited very potent inhibition. At

15 concentrations as low as approximately 30 ng/ml, each of the three anti-CD40 mAbs gave 50% of maximal inhibition. In contrast, the isotype-matched IgG2b mouse myeloma protein MOPC-141 had no effect on the immunoglobulin production.

The inhibitory effect by the three anti-CD40 mAbs was not specific for the manner of activation of the T cells providing the CD40 ligand helper activity. Figure

20 9B shows that under all the T-cell stimulation conditions (anti-CD3 alone; anti-CD3 + anti-CD2; anti-CD3 + anti-CD28; and anti-CD3 + anti-CD2 + anti-CD28), addition of the anti-CD40 mAb 5D12 results in strong inhibition of immunoglobulin production by the human B cells. The inhibition is comparable to the amount of inhibition with the hCD40.H μ fusion protein, known to completely block the CD40-

25 CD40 ligand interaction. The percentage of inhibition varied from 40 to 70% depending on the T-cell activation conditions. In contrast, the isotype-matched IgG2b mouse myeloma protein MOPC-141, or human IgM (as control for the hCD40.H μ fusion protein) had no effect on immunoglobulin production by the human B cells.

Deposition of Cultures

The hybridomas used in the above examples, to illustrate the method of the present invention were deposited in and accepted by the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, under the
5 terms of the Budapest Treaty.

	<u>Hybridoma</u>	<u>Deposit Date</u>	<u>Accession No.</u>
	B7-24	May 6, 1993	HB 11341
	3C6	May 6, 1993	HB 11340
10	5D12	May 6, 1993	HB 11339
	3A8		HB

The present invention has been described with reference to specific
embodiments. However, this application is intended to cover those changes and
15 substitutions which may be made by those skilled in the art without departing from
the spirit and the scope of the appended claims.

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